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14. ABSTRACT

The survival with Systemic Lupus Erythematosus (SLE) is closely linked to the early detection of kidney disease, making the discovery of currently elusive renal biomarkers crucial for the prognosis of SLE. A well-defined cohort of patients with SLE (n=150), disease and healthy controls were assembled and followed-for up to 1.5 years. By surface-enhanced laser desorption time to flight mass spectrometry (SELDI-TOF-MS) and matrix enhanced various TOF-MS, we discovered a set urinary lupus nephritis candidate biomarkers; namely transferrin, ceruloplasmin, alpha1-acid glycoprotein, and lipocalin-like prostaglandin-D synthetase. Urinary concentrations of these lupus nephritis candidate biomarkers differed significantly with the presence of lupus nephritis. The accuracy of these lupus nephritis biomarkers to detect a flare of disease as measured by the area under the receiver operating characteristic curve was at 0.85 excellent. Thus we characterized a novel panel of urinary markers whose preliminary study supports them being excellent biomarkers of the course of lupus nephritis.

15. SUBJECT TERMS
Proteomics, lupus nephritis, biomarkers, systemic lupus erythematosus

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INTRODUCTION

The purpose and scope of the research project is to test the following two hypotheses:

H1: Specific protein biomarkers are present in the urine and/or serum of patients with SLE nephritis that are not present in patients with SLE but without nephritis.

H2: A subset of these proteins is present in the urine and/or serum of patients with SLE prior to the onset of nephritis, and can therefore be used to predict the development of nephritis.

We propose to test these hypotheses by pursuing the following specific aims:
Specific Aim 1: To identify initial biomarker patterns in SLE nephritis using screening proteomic profiling: Initial high-throughput screening proteomic analysis will be done in Dr. Devarajan's laboratory at Cincinnati Children's Hospital Medical Center (CCHMC) on urine and serum samples using Surface-Enhanced Laser Desorption/ Ionization Time-of-Flight mass spectrometry (SELDI-TOF-MS) and the PBS-IIc ProteinChip® platform (Ciphergen Inc). The resulting spectra will be analyzed with Ciphergen Express and Biomarker Pattern Software. Changes in proteomic profiles will be confirmed and enhanced using Two Dimensional Differential In Gel Electrophoresis (2-D DIGE) and Peptidomics®. Changes in proteomic profiles will be compared to changes in currently available renal biomarkers (urinalysis, blood and urine chemistry), medications and other clinical outcomes (overall disease activity, renal and overall damage).

Specific Aim 2: To identify biomarkers predictive of SLE nephritis using advanced proteomic profiling: Advanced proteomic studies on selected sample sets will be performed at Applied Biotechnology Branch, Air Force Research Lab, Wright-Patterson Air Force Base (AFRL/HEPB), where LC/MS based protein profiling using Thermo LTQ FT-ICR will provide ultra-high resolution/mass accuracy protein identification, using the LTQ FT-ICR hybrid instrument (Thermo Electron North America LLC). The data will be analyzed by using Bioworks 3.2 software for protein identification along with statistical calculations for protein/peptide probabilities.

BODY

Overall progress made to date during this year is as follows:
All subjects with systemic lupus erythematosus SLE have been recruited. A multi-center study design is chosen to ensure timely enrollment. We have recruited approximately 150 children with SLE, including some with and some without active renal disease. We achieved our goal of recruiting 75 patients with active renal disease, 75 patients without active lupus nephritis (LN). Seventy five children with Juvenile Idiopathic Arthritis (JIA, disease controls) and 75 normal siblings of children with JIA (healthy controls) have been targeted for recruitment, and this has almost been completed. All subjects had at least five study visits to date. About 65 SLE subjects have completed all study visits.

Additionally, we will recruit up to 20 children with Focal Segmental Glomerulosclerosis (FSGS) to serve as a disease control group to better dissect mechanisms of inflammatory lupus nephritis from non inflammatory nephropathies with similar urinary findings. The respective amendment has been submitted to the CCHMC IRB and the ORP will be notified once approval at the local IRB site has been achieved.

We have analyzed clinical, laboratory, and kidney biopsy data from an initial cohort of pediatric patients with SLE (n=32). Children with juvenile idiopathic arthritis (JIA, n=11) served as controls. SELDI-TOF-MS was performed using urine samples. A consistent urinary proteomic signature for SLE nephritis was found, comprising eight biomarker proteins with peaks at m/z of 2.7, 22, 23, 44, 56, 79, 100, and 133 kDa. These biomarkers were strongly correlated with renal disease activity and with renal damage. For the diagnosis of active nephritis, the area under the curve (AUC) was $\geq$ 0.90 for these biomarkers.

More recently, we have completed the task of identifying these biomarkers. The protein peaks were gel extraction, followed by trypsin digestion and peptide mapping by SELDI-TOF-MS and/or MALDI-TOF-MS, as shown in **Table 1**.

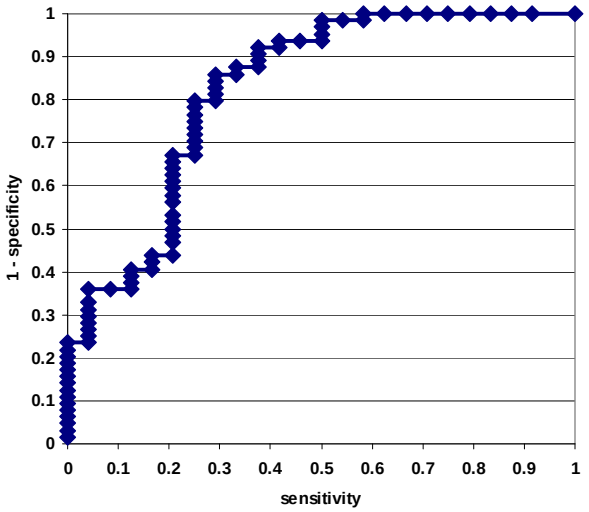
Table 1. Urinary Biomarkers for Lupus Nephritis, identified by SELDI-TOF-MS and/or MALDI-TOF-MS

2.7kDa	22kDa	23kDa	44kDa	56kDa	79kDa	100kDa	133kDa
Albumin	Albumin	Prostaglandin D synthetase	Albumin	α-1-acid glycoprotein	Transferrin	Albumin	Ceruloplasmin

In addition, our previous genomic and proteomic studies have identified NGAL as a highly promising urinary biomarker of renal disease activity and renal damage. We therefore developed and tested standardized clinical assays for each of these biomarkers. Urine samples from SLE patients were assayed for neutrophil gelatinase associated lipocalin (NGAL), lipocalin-type prostaglandin-D synthetase (L-PGDS), α1-acid-glycoprotein (AGP), transferrin (Tf), and ceruloplasmin (Cp) in addition to albumin and related fragments. In the plasma only Tf levels (p=0.02) changed with LN activity. However, with active LN, there were significantly higher urinary concentrations of L-PGDS (p=0.002), AGP (p=0.0002), Tf (<0.0001) and Cp (p=0.0003) than with inactive LN. There was a significant correlation between renal disease activity and each individual biomarker with AUC greater than 0.66. The area under the receiver operating characteristic (ROC) curve of the urinary LN biomarker panel was 0.85 for detecting active LN (**Figure 1)**

Thus, urinary concentrations of Tf, Cp, AGP, PGDS are biomarker of LN activity especially when used in combination. In our preliminary cohort, these biomarkers have excellent concurrent validity for active LN, exceeding that of any other known SLE biomarkers.

Figure 1: Receiver operating characteristic curve for the combination of urinary Tf, Cp, ACG-1 and L-PDGS



KEY RESEARCH ACCOMPLISHMENTS

- Kristina Wiers, Kathleen Haines, Marissa Klein-Gitelman, Judyann Olson, Kathleen O'Neil, Kathleen Onel, Murray Passo, Nora Singer, Lori Tucker, Shannen Nelson, Prasad Devarajan, Hermine I. Brunner. New Biological Markers for Systemic Lupus Erythematosus (SLE) Renal Disease; American College of Rheumatology 2007; Boston MA; S234.

- M. Suzuki, K. M. Wiers, M. Klein-Gitelman, K. A. Haines, J. Olson, K.B. Onel, K. O'Neil, M.H. Passo, N.G. Singer, L. Tucker, J. Ying, P. Devarajan, H.I. Brunner. Neutrophil Gelatinase Associated Lipocalin as a Biomarker of Disease Activity In Pediatric Lupus Nephritis; Pediatr Nephrol. 2008 Mar;23(3):403-12

  (See attachment)

CONCLUSIONS

Thus we characterized a novel panel of urinary markers whose preliminary study supports them being excellent biomarkers of the course of lupus nephritis.

A current problem relates to the delay in the completion of the Human Subjects Protection Review by the Army personnel. Approval of the study was obtained March 18, 2008 (HRPO Log Number: A-14216-1); CCHMC IRB number: 08-02-16.

Another problem relates to the fact that the Peptidomics® assay to be used for some of the proteomic discovery work proposed for Aim #1 is no longer commercially available because the company producing the Peptidomics® assay no longer exists. An excellent alternative approach to screen for small peptides is MS and NMR-based metabonomics. We have identified an investigator and collaborator in Ohio, Dr. Michael Kennedy, Ohio Eminent Scholar in Structural Biology, Miami University, Oxford, Ohio. His laboratory has vast experience in biomarker discovery using metabonomics. Once ORP approval has been achieved, Dr Kennedy will perform laboratory evaluations detailed in Section IV.

The following work will be completed during the next reporting period:

1. Completion of the majority of the remaining study evaluations, including collection of clinical data and biological samples.

2. Sample selection for proteomic studies at the ARFL under the leadership of Dr. Schlager. We were informed by our collaborators that a maximum of 25 assays could be run with the funds made available. Thus we will select 5 subjects of each of the groups (SLE with LN, SLE without LN, JIA patients, health controls, children with FSGS)

Metabolomic & Metabonomic Studies Of Lupus Nephritis (LN)

The assays will be performed at the laboratory of Dr. Michael Kennedy in the Eminent Scholar Laboratory in Hughes Hall at Miami University, Oxford, Ohio.
(Please note that Dr. Kennedy's CV and current support are in the Appendix 1 and facility resources are in Appendix 2).

SPECIFIC AIM:

To screen for biomarker patterns characteristic of SLE nephritis using NMR- and MS-based metabonomics.

***BACKGROUND*:**

Metabonomics: Applying metabolic profiling to differentiate between Lupus in the presence and absence of kidney disease. ***Metabolomics*** refers to global analysis of small molecule metabolites in biological fluids such as urine or serum. Identification of the resulting metabolic patterns in these fluids is regarded as ***metabolic profiling***. ***Metabonomics***, on the other hand, usually refers to measurement of changes in an organism's metabolic profile in response to external stimuli, genetic modifications, or as a consequence of presence or severity of disease. In the initial stages of searching for novel endogenous biomarkers for a human disease such as Lupus, ***non-targeted metabonomics***, which simultaneously screens all metabolites, has an advantage over ***targeted assays***, in which specific biomolecules/metabolites are selected and analyzed one (or few) at a time. Another strength of metabonomics, especially when applied to biofluids such as urine or blood, is that the corresponding metabolic profile cumulatively reflects a metabolic endpoint of cellular, tissue, and whole organism function, as well as a contribution from co-metabolism of gut flora and the ability to detect and monitor xenobiotics, all which can be assessed over a disease time-course. As a consequence, metabonomics has the potential to provide important insights into, and in-depth understanding of the underlying role that specific biochemical pathways play in the patho-physiology of various human diseases, and in particular, autoimmune diseases such as Lupus.

Over the last decade, NMR-based metabonomics has emerged as a powerful, noninvasive technique for exploring pathological metabolic and toxicological processes in humans[42-44] and is being developed as a tool for diagnosis of human diseases including inborn errors of metabolism[45, 46], investigation of the biological response to drugs for modeling drug toxicity[47], and for the study of other xenobiotics[48]. NMR-based metabonomics has certain strengths compared to its MS-based companion technique, including its relatively universal ability to detect metabolites (i.e. virtually any protonated metabolite can be detected indiscriminately using NMR methods), the rich information available about the chemical structure of the detected metabolites, the minimal sample preparation required, the superior reproducibility, and the fact that the NMR methodology is nondestructive to the samples. However, NMR also has some weaknesses compared to MS-based methodology including its relatively poor sensitivity and resolution. While MS-based metabonomics has lagged behind NMR-based metabonomics in its development and application, the number of MS-based metabonomics studies has been growing steadily over the last five years, with most applications being applied to the study of rat urine, e.g. pharmaceuticals[50, 51,] screening for biomarkers of phospholipidosis[52], heavy metal toxicity[53], and detection of strain, diurnal and gender differences[54]. While sensitivity and resolution are clear strengths of the MS-based approach, some of the disadvantages of the technique are that it requires substantial sample preparation, suffers from ion suppression when multiple metabolites co-elute from during the LC, and detection is not considered universal since only those metabolities that ionize can be detected. The current state of

development of MS-based metabonomics is summarized in several recent reviews [55, 56]. Perhaps most relevant to this application is the growing number of studies reporting the combined use of NMR- and MS-based metabonomics. These studies, which include examination of rat urine in response to mercuric chloride exposure[57], aging and development[58], and exposure to the model nephrotoxin cyclosporin A[59], indicate the beneficial and complementary nature of the combined application of NMR- and MS-based metabonomics. Comparisons of the utility of NMR- and MS-based techniques for metabonomics studies are available in recent reviews.[60, 61]

The goal of NMR- and MS-based metabonomics studies is to identify changes in metabolite concentrations in biofluids. Due to the complex nature of NMR and MS data of biological fluids, in combination with the diverse nature of patient populations, it is necessary to apply multivariate statistical analytical approaches in order to obtain meaningful, interpretable conclusions. Projection methods such as principal component analysis (PCA) are used to simplify multivariate metabonomics data to reduce the dimensionality of the data to a tractable form. In PCA, NMR spectral intensities, or LC-MS elution-m/z intensity pairs, are usually bucketed into discrete ranges prior to calculation of a covariance matrix which captures the magnitude of variation of each range across the population. Diagonalization of the covariance matrix yields a set of ***eigenvectors***, or ***principal components***, which are weighted most heavily by the buckets with the largest variation across the population, and ***eigenvalues***, which reflect the magnitude of the variation associated with the corresponding eigenvector. The weighted buckets defining the principal components are referred to as the ***"loadings"***. The first six principal components frequently account for >95% of the variance across all the spectral data. Once the critical loadings are identified, the corresponding metabolites can be identified either from database driven software or using conventional chemical analysis methods. PCA is initially implemented in an ***unsupervised*** mode to reveal if clustering is apparent in the raw data. PCA is also the foundation for developing models for classification of new, unknown samples. Initial model development is a ***supervised*** process since it requires prior knowledge of class membership of the samples in a training set. In this supervised stage, a model is developed for a normal population, or a specific disease state, and then the model is cross-validated, using the training data set, to determine its stability and reliability. If a stable, robust model can be established, it is then possible to classify new samples, typically at a goal of 95% confidence, as either belonging, or not belonging, to the model. For classification of new, unknown samples, other more sophisticated methods, such as multiple regression analysis, are implemented to maximize discrimination power. ***Discriminant function analysis (DA)*** is an example of a multiple regression approach that is widely used in NMR-based metabonomics to classify samples into different groups. This rather complicated process of data analysis relies heavily on population statistics so that models for "healthy" and "disease" populations can be established and cross-validated, and test-set classification assessed for accuracy, all indicating the necessity of a sufficient population study, as is proposed in

this application, in order to be able to assess the power of the method as it is applied to a human disease like Lupus.

EXPERIMENTAL DESIGN:

Rationale: From what we already understand about the pathology SLE and nephritis, it is reasonable to expect differences in the metabolic profiles of urine and blood obtained from SLE patients either in the presence or absence of nephritis. NMR-based metabonomics alone may not be sensitive enough to detect important signature metabolites for SLE nephritis. Therefore, we will include the more sensitive MS-based approach in order to increase the likelihood of achieving our goals in this project. By way of example, it is routinely possible to identify on the order of ~35-50 metabolites from the urine or blood using NMR-based metabonomics, whereas approximately ten times as many metabolites, or ~500, can be routinely detected or identified using the same biological samples using MS-based metabonomics. However, for reasons already discussed above, the two techniques are considered highly complementary and the most powerful metabonomics approach is considered to be the combined application of both NMR- and MS-based techniques.

Research Design: We will consider five distinct population groups as described in the proposed study flow diagram in the original application: SLE patients with or without nephritis, JIA controls, normal controls, and . Due to limited funding, we will initially apply NMR- and MS-based metabonomics analysis to just five patients from each population group, although 25 samples from each group are available. Consequently, the goal of the initial study will be to determine if metabonomics can be used to identify metabolic patterns or biomarkers to distinguish each group using a raw clustering analysis. Assuming that significant clustering is observed, it will be possible to try to determine the chemical identity the metabolites responsible for distinguishing each population group. Because of the limited population sampling in the initial study, it will not be possible to develop and validate models for each population group, however, this could be pursued if the initial data indicate that this should be possible, and given adequate future funding.

Experimental Methods: ***Urine Sample Preparation (NMR):*** 300 mL of urine will be mixed with 300 mL of phosphate buffer (25 mM phosphate, pH 7.4 in 10% D_2O, .02%w/v sodium azide) containing 0.5 mM DSS (chemical shift reference standard) and centrifuged at 13400g for 5 minutes prior to NMR analysis and stored at -20°C while not being used for NMR data collection. ***Serum Sample Preparation (NMR):*** 300 mL of sera will be mixed with 300 microliters of saline (0.9% NaCl in 10% D_2O) and centrifuged at 13400g for 5 minutes prior to NMR analysis and stored at -80°C while not being used for NMR data collection. ***Data Collection (NMR):*** NMR data will be

collected either using a Varian Inova 600 MHz spectrometer (Varian Associates, Inc) or a Bruker US^2 Avance III NMR 850 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany). Gradient shimming will be applied to each sample prior to data collection to minimize systematic variation due to field inhomogeneity. NMR data processing will be done using the Bruker Topspin software. ***NMR of urine samples:*** NMR spectra will be obtained using a simple 1D water presaturation or 1D NOESY experiments. ***NMR of serum samples:*** The Car-Purcell-Meiboom-Gill (CPMG) (RD-90°-(t-180°-t)$_n$-acquire) experiment will be used to generate proton spectra edited by T_2 relaxation. **Urine Sample Preparation (LC-MS):** 75 mL of urine will be centrifuged at 13200g for 10 min at 4C and the supernatant used directly, as demonstrated in the literature[50]. **Serum Sample Preparation (LC-MS):** 100mL of serum will be treated to remove protein components using methanol precipitation (8:1:1 methanol:water:serum), centrifuged at 13200g for 10 min at 4ºC, the supernatant collected, lyophilized, and resuspended in 95%:5% water:acetonitrile prior to injection on the HPLC. **HPLC:** Liquid chromatography will be conducted using an Agilent Technologies 1200 series HPLC, the standard instrument supplied and integrated into the Bruker MicrOTOF system[72]. Standard analytical separation techniques will be used, e.g. a Waters Corporation(Milford,MA USA) X-Bridge C-18 2.1 x 100 mm (3.5 mM) column with a 2.1x10 mm pre-column in gradient mode at 40ºC, a 0.2 mL/min flow rate, eluting components of aqueous 0.1% formic acid and acetonitrile containing 0.1% formic acid. A typical urine chromatography run will use an acetonitrile gradient from 0.1% to 25% in 40 min, to 77.4% in 60 min, to 95% in 70 min, and held at 95% for 3 min, using an equilibration time of 6 min and an injection volume of 10 mL. Total time required for a single urine analysis will be ~ 80 min. A typical serum chromatography run will use an acetonitrile gradient from 0.1% to 35% in 10 min, to 65% in 50 min, to 95% in 70 min, and held at 95% for 3 min, using an equilibration time of 6 min and an injection volume of 10 mL. Total time required for a single serum analysis will be ~ 80 min. **MS Detection:** Mass analysis will be conducted using a Bruker Daltonics (Bremen, Germany) MicrOTOF mass spectrometer equipped with an orthogonal electrospray ionization (ESI) source. Data will be collected both using positive and negative ion mode using a m/z range of 50-3000. Total ion chromatograms (TIC) will be collected for each sample. Each TIC will be calibrated using a 450 μg/mL sodium formate solution containing 0.1% formic acid to ensure mass accuracy.

NMR Data Analysis: Processed spectra will be prepared for PCA using AMIX (Bruker Biospin) by binning the data into 0.02 ppm buckets, the region from 4.5-6.0ppm excluded due to imperfect water suppression, and spectra scaled to normalized total intensity prior to PCA. Data will be subjected to blind PCA to determine if clustering patterns emerge from the raw data. The influence plot will be used to identify outliers that should be excluded. Partial least squares-discriminative analysis (PLS-DA) will be applied to maximize separation between classes. For PLS-DA the X matrix will be composed of the binned spectral data and the Y matrix will be assigned to class belonging. All of these

steps will be conducted using the AMIX software suite. Soft independent modeling of class analogy (SIMCA) as implemented in the SIMCA software package will be used to independently define PCA models and classification accuracy. The data sets will be subjected to orthogonal PLS-DA (OPLS-DA), a recent modification of PLS that removes the systematic variation of the X matrix not correlated with (i.e. orthogonal to) the response vector (the Y matrix), thus combining the attributes of PLS-DA (maximum class separation) and SIMCA (analysis of two independent PCA models). If robust models can be established for class separation, the loadings plots will be investigated to identify spectral outlier regions correlated with each disease group. If significant loadings can be identified, then conventional chemical analysis methods (LC/MS and NMR) along with comparison with spectral databases such as ChenomX will be conducted to identity metabolites of interest. ***MS Data Analysis*:** Mass spectra will be collected at each time point in the total ion chromatogram collected from each LC-MS run. Individual LC-MS analyses will be analyzed using the Bruker ProfileAnalysis software package (Bruker Daltonics). LC-MS data will be prepared for statistical analysis by a bucketing procedure analogous to that used for NMR spectra. In the case of LC-MS data, the bucket table consists of retention time (RT)-m/z pairs with corresponding intensities for each sample. Data in the bucket tables will then be analyzed using PCA inside the ProfileAnalysis software package. Identification of important loadings from the loadings plot for LC-MS data will then be achieved using the MetaboliteTools software (Bruker Daltonics). Multiple approaches will be applied to attempt to maximize class separation and to maximize classification accuracy, as described in the NMR data analysis.

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APPENDICES

1. Biosketch Dr. Michael Kennedy
2. Publication

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Michael A. Kennedy	Full Professor and Ohio Eminent Scholar

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
Muskingum College	B.S.	1984	Chemistry
University of South Carolina	Ph.D.	1989	Chemistry

Additional Training in Protein X-ray Crystallography at Synchrotron Facilities:
Brookhaven National Laboratory, RapidData2002 Course, April 21-26, 2002, Protein Crystallography
Cold Spring Harbor, Macromolecular Crystallography Course, October 8-9, 2002, Protein Crystallography
BESSY, Berlin, Germany, Protein Crystallography Workshop, October 16-29, 2002, Protein Crystallography.

A. Positions and Honors.

Positions and Employment:

1989 - 1990 Postdoctoral Scientist, University of California, San Diego, LaJolla, California
1990 - 1993 Postdoctoral Scientist, Battelle Pacific Northwest Laboratory, Richland, Washington, University of Washington, Seattle, Washington
1993 – 2000 Senior Research Scientist, Level 3, EMSL, Macromolecular Structure & Dynamics, Battelle, Pacific Northwest National Laboratory, Richland, Washington
2000 – 2001 Senior Research Scientist, Level 4, EMSL, Macromolecular Structure & Dynamics, Battelle, Pacific Northwest National Laboratory, Richland, Washington
2001 – 2006 Staff Scientist, Level 5, EMSL, Macromolecular Structure & Dynamics, Battelle, Pacific Northwest National Laboratory, Richland, Washington
2006-present Ohio Eminent Scholar in Structural Biology, Miami University, Oxford, Ohio
2006-present Full Professor, Department of Chemistry and Biochemistry, Miami University, Oxford, Ohio

Other Experience and Honors.

***Ad Hoc* reviewer for Journals:** *Journal of Structural and Functional Genomics, Journal of the American Chemistry Society, Journal of Magnetic Resonance, Biochemistry, Journal of Molecular Biology, Nucleic Acids Research, FEBS Letters, Concepts in Magnetic Resonance, Magnetic Resonance in Chemistry, Protein Science, Proteins: Structure, Function & Genetics, Nature Methods*

Ad Hoc reviewer for funding agencies:

Canadian Inst. for Health Research Genomics Review Panel: Genomics Research Program (March, 2001).
NIH Special Emphasis Panel Study Sections: 1) Structural Genomics (March 13, 2000); 2) Structural Genomics (Nov. 31-Dec. 1, 2000) 3) Structural Genomics (June 27-28, 2001).
Ad Hoc reviewer for the Department of Energy Office of Biological and Environmental Research
NIH P41 Site Visit Team, (Madison, Wisconsin, July 22-25, 2004)
National Science Foundation, Review of the Protein Data Bank, Piscataway, NJ, March, 2005.
NIH Protein Structure Initiative Special Technology Centers Review, Washington DC, April, 2005
DOE Structural Biology Center Site Visit Team, APS, Argonne National Lab, May, 2006.
NIH P01 special study section (online, November 14-15, 2007)
National Science Foundation, Major Research Instrumentation Panel, May1-2, 2008

Honors and Awards:
Distinguished Alexander Hollaender Postoctoral Fellow (1991-1993) - DOE
Northwest College and University Association for Science Fellow (1990) - DOE
Miller Research Fellow - Alternate - 1990. – U. California, Berkeley
Guy Lipscomb Award for Outstanding Research in Chemistry (1989) – U. South Carolina

PUBLICATIONS: (selected from 95 since 1989)

1. Ni, S.; Robinson, H.; Marsing, C.G.; Bussiere, D. E.; and Kennedy, M. A., Structure of 2C-Methyl-D-erythritol-2,4-cyclodiphosphate Synthase from *Shewanella oneidensis* at 1.6Å: Identification of Farnesyl Diphosphate Trapped in a Hydrophobic Cavity. *Acta Crystallographica Section D*, 60,1949-57, 2004.
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Resources - Miami University Department of Chemistry and Biochemistry

Dr. Kennedy's has ~7000 sq. ft. of laboratory space located in the Hughes Laboratories in the Department of Chemistry and Biochemistry at Miami University. The laboratory includes standard biochemistry laboratory equipment including pcr, UV-Vis, incubator shakers, French press, microscopes, -80 freezers, walk-in cold room, etc. The associated office space will support up to 6 senior investigators, 10 graduate students, and 10 undergraduate students, and contains several Linux, PC and Macintosh computers loaded with critical software for metabonomics data analysis including Bruker Amix, ChemomX, and Simca-P.

In November of 2007, Dr. Kennedy took delivery of an 850 MHz Bruker Avance III nuclear magnetic resonance spectrometer equipped with room temperature triple resonance indirect detect HCN probe with Z-axis gradient. The 850 MHz spectrometer will available as needed for this project. There will be no fee for access to this instrument for this project. The Kennedy laboratory also includes a 600 MHz Varian Inova NMR Spectrometer with triple resonance indirect detect HCN 1) room temperature and 2) cryogenic probes with Z-axis gradient and will be available as needed for this project. There will be no fee for access to this instrument for this project. The Kennedy laboratory also has a HPLC-MS system that will be available as needed for this project. The HPLC-MS system is equipped with an Agilent 1200 series HPLC and a Bruker Daltonics MicrOTOF mass spectrometer. The system has all of the latest software for mass-spectrometry based metabonomics analysis. There will be no fee for access to this instrument for this project.

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ORIGINAL ARTICLE

Neutrophil gelatinase-associated lipocalin as a biomarker of disease activity in pediatric lupus nephritis

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Abstract We hypothesized that neutrophil gelatinase-associated lipocalin (NGAL) is an early predictive biomarker of disease activity in lupus nephritis. NGAL in serial plasma (PNGAL) and urine (UNGAL) samples was measured by enzyme-linked immunosorbent assay (ELISA) in 85 participants with pediatric systemic lupus erythematosus (pSLE), healthy children (n=50), and children with juvenile idiopathic arthritis (JIA) (n=30). Disease activity was measured by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI). Plasma and urinary NGAL were significantly increased in subjects with pSLE compared with those with JIA or with healthy controls (all $p<0.03$), and unrelated to subjects' age, weight, or height. Plasma and urinary NGAL were stable in pSLE subjects with unchanged disease activity. The pSLE subjects with worsening global or renal disease activity had a mean ± standard error (SE) increase of UNGAL (in ng/ml) of 11.5± 6.4 or 36.6±12.1 ($p<0.01$), corresponding to a 156% or 380% increase, respectively. PNGAL increased with worsening disease but to a much lesser degree than UNGAL [global disease activity (mean ± SE): 7.3±6.2 or 21%; renal disease activity: 20.2±6.0 or 51%; both p=not significant]. In conclusion, NGAL in urine but not in plasma represents a novel biomarker for renal disease activity in pSLE.

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Introduction

Renal involvement is one of the main determinants of poor prognosis of systemic lupus erythematosus (SLE) [1] and is more frequently encountered in children than in adults with SLE. Currently available renal biomarkers, i.e. measures of the degree of SLE renal disease activity and severity, are too insensitive to allow for early identification of patients with active SLE nephritis, prohibiting timely initiation of therapy to avoid permanent renal damage [2]. Randomized clinical trials in SLE are hindered by the lack of high-quality biomarkers to verify the effects of therapies within a short period of time [3].

Neutrophil gelatinase-associated lipocalin (NGAL) is a member of the lipocalin family of proteins that has been extensively studied in acute kidney injury [4]. NGAL is one of the most robustly expressed proteins in the kidney following ischemic or nephrotoxic injury in both animals [5–9] and humans [10–13]. Importantly, a recent prospective pediatric study demonstrated that concentrations of NGAL in urine and plasma represent novel, sensitive, and specific biomarkers for early identification of acute kidney injury following cardiac surgery [13]. Our previous preliminary data also suggest that urinary NGAL levels are markers of renal disease activity and renal damage in children with pediatric SLE (pSLE) [14].

Plasma NGAL concentrations are elevated in patients with atherosclerosis, ovarian cancer, and systemic vasculitis, including Kawasaki syndrome [15–18], whereas plasma NGAL levels in pSLE have not been investigated. In this study, we hypothesized that both urinary and plasma NGAL change with renal disease activity. The purpose of this study, therefore, was to assess the relationship of urine and plasma NGAL levels with disease activity in pSLE with a special emphasis on nephritis.

Methods

Patients

With approval of the institutional review boards of the participating institutions, children fulfilling American College of Rheumatology Classification Criteria for SLE [1] prior to the age of 16 years were studied during routine visits to the pediatric rheumatology and lupus clinics. A convenience sample of 30 children diagnosed with juvenile idiopathic arthritis (JIA) [19] were recruited as disease controls. Samples of healthy controls (n=50) were obtained from the Cincinnati Genomic Control Cohort assembled by the Cincinnati Children's Hospital Medical Center.

Study design

The medical record was reviewed to screen for preexisting renal disease in subjects with JIA and to obtain pSLE-specific information. Review of system information and the results of routine laboratory testing at the time of the study visits were recorded. Relevant demographic data of all participants were obtained, as was information on medication regimens. NGAL levels were assessed cross-sectionally in all subjects (SLE subjects, JIA controls, healthy controls) and over time in the 52 pSLE subjects with available NGAL testing over time in 3 month intervals.

Laboratory testing for NGAL

NGAL levels in urine and plasma were quantified by enzyme-linked immunosorbent assay (ELISA) using an NGAL ELISA kit (Kit 036; AntibodyShop, Grusbakken, Denmark) that specifically detects human NGAL. The assay was performed as per the manufacturer's protocol. Briefly, 100 μl of NGAL standards or diluted samples (urine or plasma) were applied to the precoated microwells in duplicates. Microwells were then incubated for 1 h at room temperature and then washed with washing buffer. In succession, biotinylated NGAL antibody and horseradish peroxidase (HRP)-streptavidin were incubated in the wells for 1 h each with shaking at 200 rpm. Tetramethylbenzidine dihydrochloride (TMB) substrate was added for 10 min in the dark before adding stop solution. Finally, NGAL concentration was measured at 450 nm wavelength in each well, with reference reading at 620 nm in blank wells. The intra-assay coefficients of variation were 2.1% (range: 1.3–4.0%) and 3.0% (range: 1.2–4.0%) in urine and plasma, respectively. Interassay variation was 9.1% (range: 6.8–18.1%) and 8.2% (range: 2.2–11.2%) in urine and plasma, respectively. Urine creatinine was measured using quantitative colorimetric Microplate Assay Kit (Oxford Biomedical Research, Oxford, MI, USA) to standardize urinary NGAL for changes in urine concentration. Urinary NGAL (UNGAL) excretion is presented as the amount of urinary NGAL in nanograms per milliliter (ng/ml) urine (UNGAL-ml), as well as urinary NGAL in nanograms per milligram (ng/mg) of urine creatinine to correct for differences in NGAL due to urine dilution (UNGAL-crea). The concentration of NGAL in the plasma (in ng/ml plasma) is referred to as PNGAL. All measurements were made in triplicate and in a blinded fashion.

Information obtained for pSLE subjects

Changes in global disease activity were measured by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI-2K) [20]. Extrarenal disease activity was defined as the summary score of the SLEDAI-2K excluding the scores accrued in the renal domain of the SLEDAI-2K. Renal disease activity was defined as the sum of the SLEDAI-2K scores accrued in the renal domain of the measurement. Higher SLEDAI-2K scores represent more active disease.

All participating centers perform kidney biopsies on any pSLE patient who has abnormal urinalyses that cannot be explained by mechanisms other than SLE. Because some renal biopsies were obtained prior to the introduction of the new classification system of SLE nephritis [21], the original system was used [22].

Laboratory testing recorded for the study included blood urea nitrogen (BUN), serum creatinine, urinalysis and urine microscopy, urinary protein to creatinine ratio, serum complement levels C3 and C4 (categorized as normal or low), hemoglobin, erythrocyte sedimentation rate (ESR), and titers of anti-dsDNA antibodies (Crithidia or Farr assay; categorized as negative or positive/elevated).

Worsening (flare) of overall (renal) pSLE disease course between visits was measured in two ways. First, we recorded physician-rated worsening of global (renal) disease as defined by an increase in the scores of the disease activity estimate on a physician visual analog scale (MD-VAS; range 0–10). Second, we determined any increase in the scores of the overall (renal domain) score of the SLEDAI-2K. Global disease activity was considered unchanged or improved in cases where the MD-VAS or the SLEDAI-2K suggested that the pSLE subject's disease was stable or improved, respectively. Additionally, physicians completed a Likert scale to indicate whether there were global (renal) flares, stable global (renal) disease, or improving global (renal) disease between study visits.

Control population

Plasma and urine specimens from healthy controls were generously donated by the Cincinnati Genomic Control Cohort. Information on the review of systems as well as demographic information was available. For participants with JIA, the results of routine urinalyses and serum creatinine testing ordered to screen for nonsteroidal anti-inflammatory drug (NSAID)- and/or methotrexate-related toxicity were recorded. In addition, information pertaining to subject demographics and the JIA core response variables was obtained [23], including ESR, physician-rated disease activity (VAS 0–10), and the number of joints with active arthritis and those with limited range of motion.

Data analysis

EXCEL XP (Microsoft Inc., Redmond, WA, USA) and SAS 9.1 (SAS Institute Inc., Cary, NC, USA) were used for analysis. Means and standard errors (SE) values were calculated as measures of central tendency. Groups of patients were assessed for statistically significant differences using analysis of variance (ANOVA). For pSLE subjects, plasma and urinary NGAL levels, the values of laboratory parameters (serum creatinine, glomerular filtration rate, proteinuria, urinary protein to creatinine ratio, titers of anti-dsDNA antibodies, hemoglobin), and scores of disease measures [SLEDAI-2K, British Isles Lupus Activity Group (BILAG) index, Systemic Lupus International (SDI)] were correlated using Pearson's correlation coefficients (r). Mixed models correcting for differences in gender and race were used to assess changes of NGAL for important differences over time in the subset of cSLE subjects with available longitudinal data. Post hoc testing was performed with the Tukey procedure.

Results

Pediatric SLE subjects

Data of 85 subjects with pSLE were available, and 52 of them had at least one follow-up visit (total number of visits: 132). The demographic information of pSLE subjects is summarized in Table 1 and results of their laboratory testing are shown in Table 2. The mean time ± SE between the first study visit and the time of renal biopsy was 2±0.35 years for those subjects who had biopsies (n=48).

At baseline, the mean±SE of PNGAL, UNGAL-ml, and UNGAL-crea of pSLE subjects was 63.6±4.7, 44.6±7.3, and 29.2±4.6, respectively. UNGAL was unrelated to PNGAL (r<0.13; p=NS), whereas UNGAL-ml was strongly correlated with UNGAL-crea (r=0.8; p<0.0001).

JIA controls

Thirty children with JIA (female:male=27:3; mean ± SE age 15.6±0.1 years) participated in the study. There were four African American and 26 Caucasian subjects with JIA. These subjects were treated with NSAIDs alone (n=2), methotrexate (MTX) alone (n=8), or the combination of NSAIDs and MTX (n=12). Eleven JIA subjects were treated with biologic medications (etanercept, abatacept, infliximab, adalimumab) alone or in combination with NSAIDs and/or MTX. The mean ± SE number of active joints and joints with limited range of motion was 2.3±0.3 and 1.7±0.1, respectively; ESR or C-reactive protein (CRP) levels were elevated in seven (25%) of the 24 JIA controls

Table 1 Demographics and disease outcomes of subjects with pediatric systemic lupus erythematosus (pSLE)

	Parameter	Number	Percent of total	Mean (SE)
Gender (female:male)	72:13	85	85: 15	
Race	American Indian	3	4	
	Asian	11	13	
	African American	27	32	
	Pacific Islander	1	1	
	Caucasian	42	49	
	Other	1	1	
Ethnicity	Hispanic	9	11	
	Non-Hispanic	76	89	
Age (in years)				15.5 (0.52)
Disease duration (in years)				5.8 (1.76)
Current medications	Prednisone	65	76	17 mg/day (1.9)
	Hydroxychloroquine	70	82	
	Azathioprine, mycophenolate mofetil	47	55	
	Cyclophosphamide[a]	21	25	
	Aspirin, NSAIDs	19	22	
	Angiotensin-blocking agents	29	34	
Renal biopsies	None available[b]	37		
	WHO class II	1		
	WHO class III	8		
	WHO class IV[c]	23		
	WHO class V	16		
	Time since renal biopsy (in years)			2 (0.35)

SE standard error, *NSAIDs* nonsteroidal anti-inflammatory drugs, *WHO* World Health Organization

[a] Five subjects were treated with cyclophosphamide at the time of study enrollment

[b] Two subjects with transient renal disease as per treating physician did not have a renal biopsy

[c] The biopsies of three patients showed considerable overlap of WHO IV and WHO V nephritis

with available data. None of the JIA controls had a history of chronic or recent acute urinary tract infection, and all had normal urinalyses and normal serum creatinine levels.

The mean±SE of PNGAL, UNGAL-ml, and UNGAL-crea were 60.7±9.7, 24±3.8, and 17.5±3.1, respectively. There was no apparent relationship between UNGAL and PNGAL in JIA ($r<0.1$; p=NS). PNGAL and UNGAL (UNGAL-ml or UNGAL-crea) were unrelated to physician-rated JIA disease activity (MD-VAS with range 0–10) and the weekly dose of MTX. Furthermore, NGAL in plasma or urine did not differ with exposure to NSAIDs or biologic medications.

Healthy controls

The 50 healthy children (female:male=28:22) had a mean± SE age of 14.8±0.05 years. There were 16 African American and 34 Caucasian healthy controls. The mean±SE of PNGAL, UNGAL-ml, and UNGAL-crea were 71.4±0.6, 15±0.4, and 7.9±0.2, respectively.

Cross-sectional differences in NGAL levels between pSLE subjects and controls

Using ANOVA and Tukey post hoc testing, differences in NGAL levels between groups of subjects (pSLE, JIA subjects, and healthy children) were assessed. UNGAL did not differ significantly between JIA and healthy controls. Conversely, pSLE subjects had significantly higher UNGAL-ml ($p<0.003$) and UNGAL-crea than did controls ($p<0.0001$). With respect to PNGAL, JIA controls had significantly higher levels than did healthy controls ($p<0.03$), and pSLE subjects had significantly higher levels than did subjects with JIA ($p<0.03$). Irrespective of diagnosis (JIA, pSLE, or healthy), NGAL (urine or plasma) did not differ with patient weight, height, or age. Among pSLE subjects, Caucasians had a trend toward lower NGAL (urine and plasma); the same was true for male compared with female pSLE subjects (p=NS). UNGAL but not PNGAL correlated with the blood pressure of JIA and pSLE subjects (all $r>0.32$; $p<0.002$).

NGAL: relationship to pSLE disease features

Correlation of NGAL with select pSLE laboratory parameters and treatments

NGAL (urine and plasma) was unrelated to the daily dose of prednisone, creatinine clearance, complement C3 and C4 levels, and ESR (all $r<0.2$). There was a significant correlation between the urine protein:creatinine ratio and PNGAL (Spearman correlation coefficient $r^2=0.2$; $p=0.03$), UNGAL-ml ($r^2=0.3$, $p=0.003$), and UNGAL-crea ($r^2=$

Table 2 Disease measures and laboratory testing results of subjects with pediatric systemic lupus erythamutosus (pSLE)

SLE disease parameter	Number	Mean (SE)	Definition of presence of abnormal value	Number (%) of tested patients with abnormal values
Laboratory measures				
Erythrocyte sedimentation rate	68	23 (2.5)	Increased level of ESR	48 (70)
Serum creatinine	85		Increased serum creatinine level of age or weight	3 (4)
Serum complement C3 or C4	85		Decreased level of C3 and/or C4	46 (54)
Anti-ds-DNA antibodies levels	57		Elevated levels of anti-dsDNA antibodies (Crithidia)	50 (88)
Protein/creatinine ratio	85	0.93 (0.26)	Urine protein/creatinine >0.2	28 (33)
Hematuria	85		At least 5 RBC/HPF	14 (16)
Pyuria	85		At least 5 WBC/HPF	13 (15)
Granular or hemegranular casts	85		At least 1 cast/HPF	15 (18)
Disease Indices				
Global disease activity	85	5.3 (0.55)	SLEDAI score >0	
Renal disease activity SLEDAI	85	2.1 (0.42)	Renal domain SLEDAI score >0	26 (31)
Extrarenal disease activity – SLEDAI	85	3.21 (0.32)	Extrarenal domain SLEDAI score >0	65 (76)
Global disease damage (SDI)	85	0.56 (0.13)	SDI >0	25 (29)
Extrarenal damage	85	0.05 (0.02)	Extrarenal domain SDI score >0	23 (27)
Renal damage	85	0.05 (0.02)	Renal domain SDI score >0	4 (5)
MD assessment of global disease activity	85	2.34 (0.19)	Visual analog scale >0 ; range 0–10	13 (15)
MD assessment of renal disease activity	85	5.44 (0.51)	Visual analog scale >0; range 0–10	19 (22)

SE standard error, *SDI* Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index, *MD* physician, *ESR* erythrocyte sedimentation rate, *RBC* red blood cells, *WBC* white blood cells, *HPF* high-power field, *SLEDAI* Systemic Lupus Erythematosus Disease Activity Index, version 2 k

0.43, $p=0.001$). NGAL (urine and plasma) levels did not significantly differ with the use of immunosuppressive medications. There was a trend toward higher UNGAL in subjects treated with angiotensin-blocking drugs (for UNGAL-crea: 42±8.2 vs. 24±5.4; p=NS). Among the laboratory indicators tested, PNGAL was only weakly correlated to the pSLE subjects' ESR ($r=0.22$; $p=0.06$).

Plasma NGAL and disease activity

The pSLE subjects with inactive disease (SLEDAI-2K=0) had somewhat lower PNGAL than those with active disease (54.5±10.3 vs. 65.3±5.1; p=NS). PNGAL did not differ significantly between pSLE subjects with vs. without active renal disease activity (62.5±7.3 vs. 64.1±5.9; p=NS). There was a trend towards higher PNGAL with active vs. inactive extrarenal disease activity (65.9±5.3 vs. 56.1±9.5; p=NS).

Urinary NGAL and disease activity

UNGAL correlated moderately with renal disease activity as measured by the SLEDAI-2K (for UNGAL-crea: $r=0.4$; $p<0.008$). The mean±SE of UNGAL-crea was 45.4±11.6 with active renal disease activity and only 21.7±3.7 in subjects with inactive lupus nephritis ($p=0.02$); the mean± SE of UNGAL-crea differed with active vs. inactive global disease activity (SLEDAI >0 vs. SLEDAI = 0; 31.7±5.2 vs. 15.6±7.3; $p=0.06$). UNGAL-crea did not change with extrarenal disease activity (active vs. inactive extrarenal SLEDAI >0 vs. = 0: 65.3±5.1 vs. 54.5±7.3; $p=0.2$).

NGAL and findings on renal biopsy

Renal damage as measured by the SDI was present in only four subjects, hence rare in this cohort. UNGAL-crea but not UNGAL-ml or PNGAL differed between groups of pSLE subjects with various degrees of renal involvement [based on World Health Organization (WHO) class] when compared by ANOVA ($p<0.02$). When analyzing only the 12 subjects whose kidney biopsy was performed within 2 months of NGAL measurement, the NGAL values differed significantly between subjects with WHO class IV vs. class V lupus nephritis. The mean (SE) of PNGAL, UNGAL-ml, and UNGAL-crea with WHO class IV lupus nephritis ($n=7$) was 95 (19), 60 (27), and 58 (17) respectively, compared with subjects with class V lupus nephritis ($n=5$) with corresponding values of 49 (11), 16 (4), and 10 (3),

respectively. There was a statistically significant difference in UNGAL-crea between class IV and class V by nonparametric ANOVA (p=0.02).

Relation of NGAL with worsening in disease activity over time in pSLE

Changes of PNGAL in relation to worsening disease activity are summarized in Fig. 1 and those of UNGAL are shown in Fig. 2 using UNGAL-ml as an example (as UNGAL-crea showed similar relationships to changes in disease activity).

PNGAL and worsening of pSLE

Similar trends were observed for both relative (% change of PNGAL) and absolute changes of PNGAL over time. PNGAL increased particularly with worsening renal disease activity (MD-rated, SLEDAI-renal). For example, PNGAL increased by 40% or a mean±SE (mg/ml) 51±19.4 with worsening SLEDAI-renal scores. Irrespective of the external standard considered (MD-VAS, SLEDAI-2K), none of these PNGAL changes reached statistical significance (Fig. 1).

UNGAL and worsening of pSLE

UNGAL levels often increased significantly with worsening of global disease activity but particularly increased with worsening renal disease activity (Fig. 2), irrespective of the external standard used (MD-VAS, SLEDAI-2K). For example, with increasing renal disease activity (renal SLEDAI-2K) mean±SE of UNGAL-ml and UNGAL-crea rose by 380% and 125% or 27±12 and 9±8, respectively (all p<0.01).

NGAL and physician-rated clinically significant changes in renal disease

A Likert scale was completed by the treating physician to indicate whether pSLE subjects' renal disease had im-

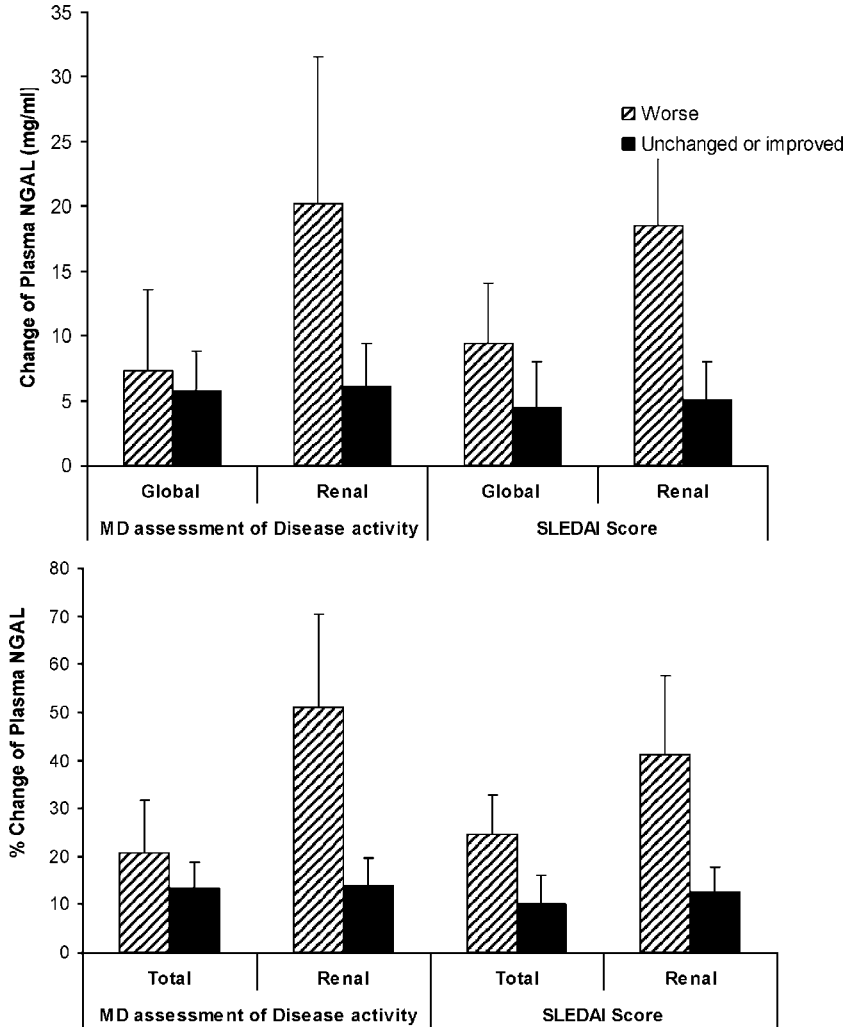

Fig. 1 Mean±standard error (SE) of absolute changes in plasma neutrophil gelatinase-associated lipocalin (NGAL) are depicted in the *upper panel* and the mean±SE of percentage changes of plasma NGAL are shown on the *lower panel* with changes in global and renal disease activity. Plasma NGAL increased with worsening of global but less pronounced as with worsening of renal disease activity. Increases in plasma NGAL varied widely, and after correction for differences in NGAL levels for race and gender in mixed model analysis, none of the changes reached statistical significance at p<0.05 in mixed-model analysis. Also see legend, Table 2

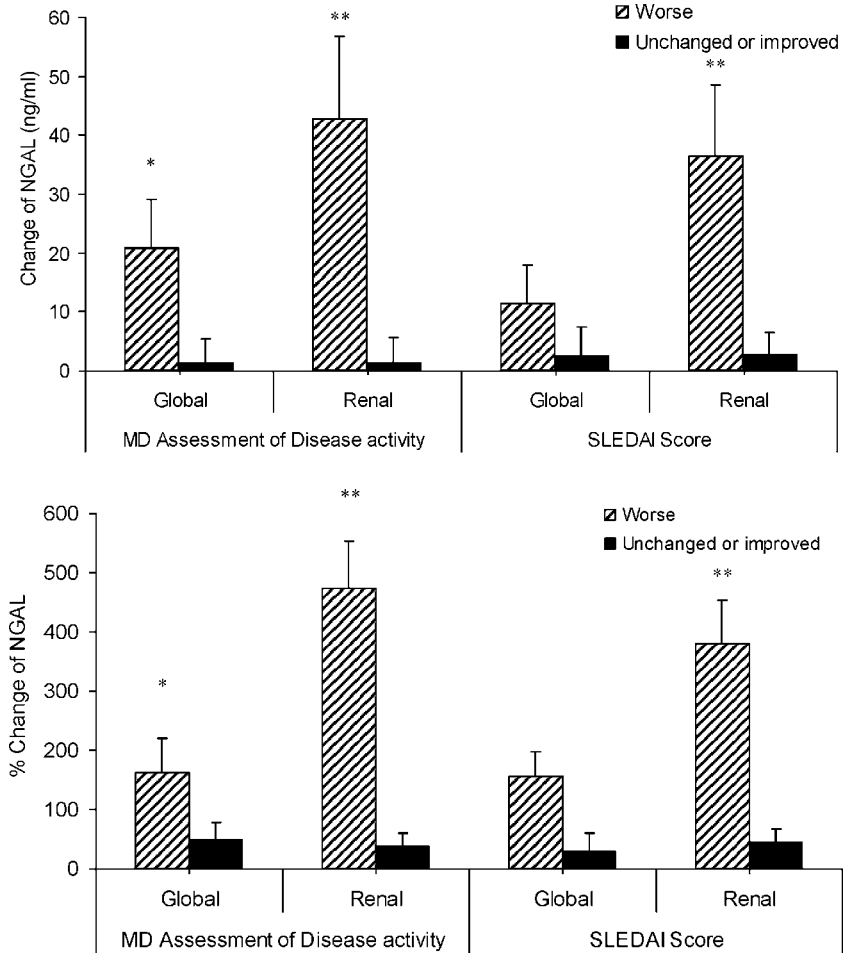

Fig. 2 Mean±standard error (SE) of absolute levels of neutrophil gelatinase-associated lipocalin (NGAL) (ng/ml urine) in the urine increased significantly with worsening of renal disease activity ($p<0.01$), irrespective of the external standard [physician visual analog scale (MD-VAS) assessment or renal domain score of the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI-2k)] chosen (*upper panel*). The same was true when the mean±SE of relative changes (%) were considered (*lower panel*). Although urinary NGAL increased with worsening of global or overall disease activity, such changes were less pronounced and only reached statistical significance when the MD global assessment was used as external standard. Also see Table 2 legend; * $p<0.05$ based on mixed models correcting for race, gender; ** $p<0.01$ based on mixed models correcting for race, gender

proved ($n=16$), worsened ($n=8$), or was stable ($n=17$) between visits. The mean±SE of PNGAL decreased by 10.6±2.0 with renal improvement, decreased by 22.6±11 with renal flare, and increased by 4.9±0.7 when renal disease was considered to be stable. The respective changes of UNGAL-ml were −56.8±6.0, +26.8±12.3, and −14.9±0.5; and of UNGAL-crea −51.5±6.2, +7±7.7, and −5.6±0.5. Figure 3 provides a comparison of the relative (%) changes of NGAL in plasma and urine with changes in renal disease activity as PNGAL remained relatively stable despite changes in renal disease. This is different from changes in UNGAL-ml and UNGAL-crea, which increased by 340% and 129% with renal flares, whereas with improving renal disease, UNGAL-ml and UNGAL-crea decreases of 47% and 10% were observed, respectively. On average, only small increases of UNGAL occurred in pSLE subjects whose renal disease was considered unchanged. Differences in UNGAL-ml and UNGAL-crea, but not those of PNGAL, reached statistical significance at $p<0.01$ and $p<0.05$, respectively.

Changes in other renal disease measures over time

Complement levels (C3, C4) and the protein:creatinine ratio changed with disease course but to a lesser degree, as shown in Table 3.

Discussion

Our cross-sectional and longitudinal data indicate that urinary NGAL rather than plasma NGAL is closely related to worsening global or renal disease activity in pSLE. Irrespective of whether urinary NGAL excretion was standardized for urinary creatinine excretion (UNGAL-crea) or not (UNGAL-ml), worsening renal disease activity resulted in marked increases of UNGAL. Plasma concentrations of NGAL fluctuated widely in pSLE, and there was no significant increase with renal disease activity change.

Our previous research supports that UNGAL may be a new sensitive biomarker of lupus nephritis, with increased

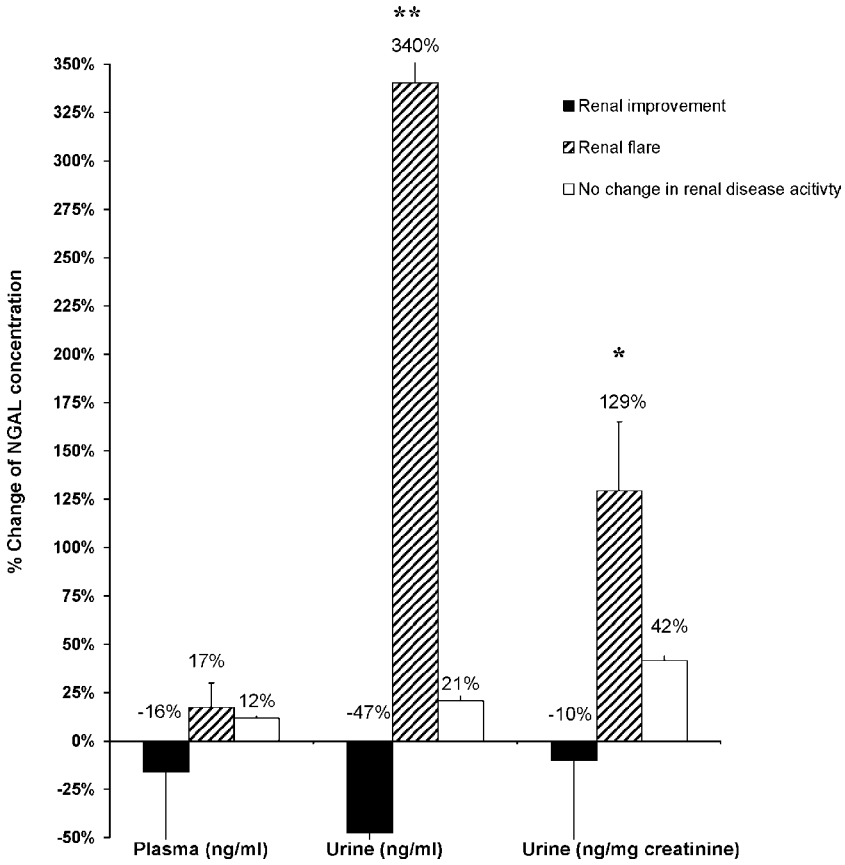

Fig. 3 Mean±standard error (SE) of relative (%) changes in neutrophil gelatinase-associated lipocalin (NGAL) with changes of lupus nephritis as judged by the treating physician are depicted for (1) plasma NGAL, (2) urinary NGAL, and (3) urinary NGAL corrected for urinary creatinine. Mean±SE of changes of NGAL in plasma were small compared with those of NGAL excreted in urine and did not reach statistical significance. * $p<0.05$ based on mixed models correcting for race, gender; ** $p<0.01$ based on mixed models correcting for race, gender

levels present in both patients with active lupus nephritis or renal damage due to pSLE. In the current cohort with its relative short disease duration of 5.8 years of subjects with renal biopsy-proven lupus nephritis, disease damage was rare, prohibiting a statistical analysis of the relationship of disease damage and NGAL. As in our previous study, UNGAL was related to disease damage in pSLE [14]. This is supported by a recent publication by Pitashny et al. who correlated UNGAL in adult SLE patients with renal parameters of disease activity and severity [24].

UNGAL elevations have been noted with several other renal diseases and are not specific for pSLE [25–27]. We speculate that UNGAL in pSLE nephritis is produced principally by the injured tubule cells, in direct proportion to the degree and severity of disease. Our studies do not rule out contributions from other cell types, such as

Table 3 Changes of complement levels and urine protein:creatinine ratio over time[a]

	Type of change	% (SE) change of complement[c]		Absolute change (SE) in protein:creatinine ratio
		C3	C4	
MD-rated change in global disease activity	Worse	+7% (2%)	+15% (9%)	−0.06 (0.04)
	Better or same	+6% (10%)	+26% (9%)	−0.23 (0.06)
MD-rated change in renal disease activity	Worse	−4% (7%)	−15% (10%)	+1.6 (0.15) [b]
	Better or same	+28% (3%)	+48% (4%)	−0.61 (0.05) [b]
Total SLEDAI score between visits	Worse	0% (1%)	+30% (5%)	+0.1 (0.22)
	Better or same	+45% (6%)	+56% (6%) [b]	−0.25 (0.08)
Renal SLEDAI scores between visits	Worse	+7% (4%)	−10% (15%)	+0.1 (0.12)
	Better or same	+13% (1%)	+34% (2%)	−0.55 (0.05) [b]

MD physician, *SLEDAI* Systemic Lupus Erythematosus Disease Activity Index, *SE* standard error

[a] For legend please see Table 1

[b] $p<0.05$ in mixed models correcting for race and gender

[c] Complements were not measured by a central laboratory. Thus different ranges of normal were present, making % changes the more relevant group comparator

neutrophils [15] or inflamed vasculature [28], as sources of UNGAL in pSLE. However, the fact that UNGAL excretion levels correlated with the markers of renal disease activity as well as changes in renal disease activity and involvement much stronger than with global disease activity as well as the changes in global disease activity, suggests that the renal epithelial cells are the major source of NGAL detected in urine. In addition, NGAL produced elsewhere in the body is thought to be almost completely reabsorbed by the kidneys at the level of the proximal tubule, unless there is concomitant renal injury [13, 29]. This is supported by the observation that PNGAL and UNGAL levels correlated only weakly with each other in pSLE. Additional support is provided by the observation that healthy controls and children diagnosed with JIA who, despite requiring anti-inflammatory and potentially nephrotoxic medications, have very low UNGAL levels.

Our data support previous reports that NGAL levels do not differ with patient age, gender, or race [13, 24, 29]. The trend toward higher NGAL levels among African Americans may be due to their higher prevalence of nephritis compared with the participating Caucasian patients with pSLE (28/37=75% vs. 22/42=52%; p=NS).

Previous studies have indicated that UNGAL is a predictive biomarker for acute kidney injury. UNGAL measured at 2 h after an ischemic insult are 98% sensitive and 100% specific in predicting acute kidney injury up to several days later [13]. Although our results provide firm support of NGAL changes with worsening of lupus nephritis, and our previous studies support that UNGAL is very sensitive and specific for identifying pSLE patients with biopsy-proven nephritis, active renal disease and renal damage [14], the paucity of longitudinal data did not allow us to effectively test for the predictive properties of NGAL in pSLE.

In exploratory analysis, PNGAL levels were markedly and significantly increased with neurological and vascular disease activity as measured by the BILAG index [30]. However, this was based on few observations and not the primary focus of our research. Of note, renal-specific changes of NGAL persisted irrespective of whether the SLEDAI-2K or the BILAG index was used to measure pSLE disease activity.

NGAL excretion in relationship to cyclophosphamide therapy requires further investigation, as alkylating agents are known to cause uroepithelial injury. However, corrected for renal disease activity, NGAL levels of the five subjects treated with cyclophosphamide at the time of the NGAL measurement did not differ from those of pSLE subjects who previously received cyclophosphamide or had never been exposed to the drug (data not shown).

A limitation of this study may be that renal biopsies were often not obtained in close timely relationship to the study, limiting their suitability to serve as an external standard for NGAL validation. This is because renal histology can change rapidly with therapy, and current laboratory markers are not suitable to accurately estimate the degree of lupus nephritis. However, in the limited number of subjects examined with kidney biopsies obtained within 2 months of NGAL measurement, the UNGAL levels appeared to discriminate between WHO class IV and class V lupus nephritis, UNGAL levels being significantly greater in subjects with class IV disease. However, this finding needs to be confirmed in larger studies.

Another limitation is that additional validation studies based on larger longitudinal data sets are required to construct receiver operating characteristic curves in an effort to specify clinically relevant changes of UNGAL that are indicative for SLE renal flares. Especially important will be to examine the predictive properties of NGAL, e.g. to delineate whether rises of UNGAL are indicative of future renal flares, and whether urinary NGAL levels rise before C3 levels fall or before serum creatinine or proteinuria increase.

In summary, we present evidence that UNGAL represents a high-quality biomarker for SLE renal disease. Although these data have been obtained in young patients with pSLE, NGAL measurement may also be useful for older adults with SLE given the similarities in the underlying disease processes of both pSLE and adult-onset SLE. Additional research is required to further characterize the measurement properties of UNGAL in children and adults with SLE.

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